

pH Zone-Refining Countercurrent Chromatography for Resolution of Tropane Alkaloids and Applications for Preparative Scale Separations

Timothy J. Waybright and Thomas G. McCloud

Natural Products Support Group, SAIC-Frederick, Inc., National Cancer Institute at Frederick, Frederick, MD

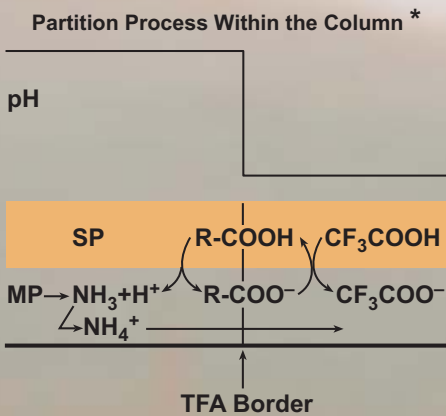
Introduction

Liquid/liquid partitioning systems have long been used as a technique to obtain alkaloid-enriched fractions from crude natural product extracts. Conventional HPLC methods are then used to further resolve and isolate the alkaloids. When substantial quantities of alkaloids must be purified, the loading capacity of the HPLC column becomes a serious limitation. In a high-speed countercurrent chromatography apparatus of the Ito type, partition coefficient changes of alkaloids brought about by changes in the pH of the phases can be exploited to achieve resolution. This technique has been termed ‘pH Zone Refining Countercurrent Chromatography’ and was used to separate a mixture of several tropane alkaloids.

pH Zone Refining Countercurrent Chromatography

Countercurrent chromatography is a gentle form of chromatography that relies upon the inherent immiscibility of certain solvents and the preference of molecules to favor and selectively partition into one phase or layer over another. It has been used for analytical and preparative separations of many different classes of compounds. In pH zone-refining methods, as with regular countercurrent methods, a two-phase solvent system is mixed and thoroughly equilibrated. The layers are separated, one layer acidified, and one is basified. Depending upon the requirements, the acidic (or basic) stationary phase layer is pumped into the coiled tubular column (in this case an ITO High Speed CCC apparatus), the column rapidly rotated, and the basic (or acidic) layer is introduced and passes over the stationary phase (Figure 1).

Figure 1



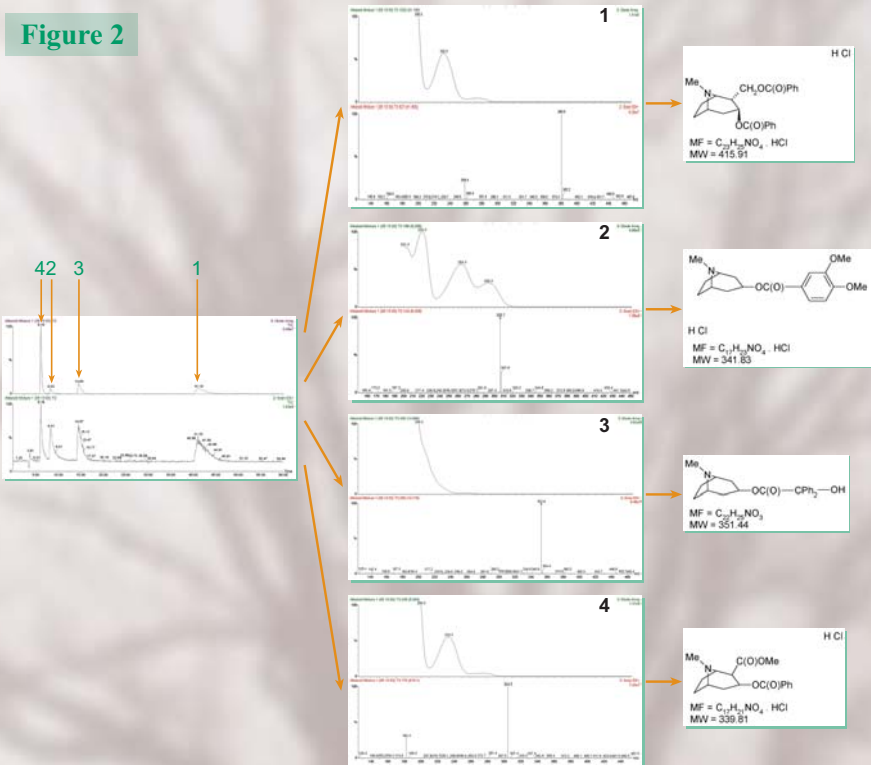
* Taken from "High Speed Countercurrent Chromatography, edited by Yoichiro Ito and Walter D. Conway, John Wiley & Sons, Inc., New York, NY, 1996."

The planetary motion of the coil allows the two phases to mix, and the centrifugal force keeps the stationary phase in place. The interaction of the two layers, and the constant change in the acid/base equilibrium as the mobile phase front passes through the column provides an environment where protonation and deprotonation occur. This zone of interaction moves slower than the mobile phase, resulting in an abrupt change in pH as this zone elutes from the coil. Separation of closely related molecules can be achieved by utilizing this difference in the mobile phase front and the pH front.

Experimental

Four different tropane alkaloids were procured from the NCI repository. Their identity was confirmed by the presence of the correct mass ion via HPLC/MS (Figure 2).

Figure 2



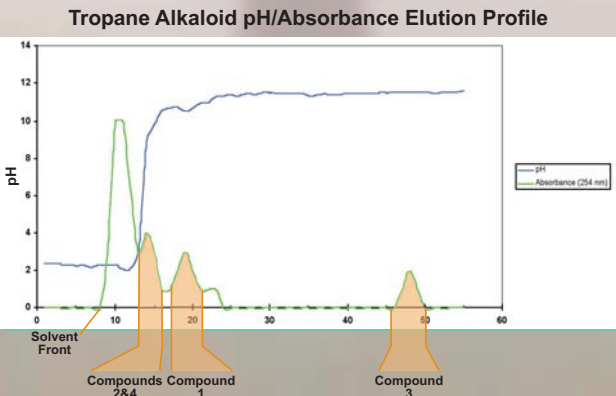
A two-phase solvent system of Chloroform/Methanol/Water (5:10:6) was thoroughly mixed and allowed to equilibrate overnight. Immediately prior to use, the layers were separated. To the lower layer (Stationary Phase) was added 0.1ml Trifluoroacetic Acid (pH ~ 2.5). 2 ml of 5.0 M ammonium hydroxide was added to the upper layer (Mobile Phase) with a pH ~ 11. The Ito coil was filled with the lower SP and rotated at 750 rpm.

Compounds 1, 2, 3 (5 mg each), and 4 (0.5 mg) were mixed and dissolved in 1.5 ml each of the basic MP and non-acidic SP. The mobile phase flow was started and the sample injected. Fraction collection (5 minutes each) began immediately after injection.

Discussion

As seen in Figure 3, the alkaloids eluted in three major zones. Samples 2 and 4 were eluted with the pH front in fractions 14-15, while Sample 1 came out in fraction 19-20 and Sample 3 eluted at fraction 49, as determined by MS/FIA (data not shown). The pH profile shows the solvent front eluting first, followed by the pH-change border.

Figure 3



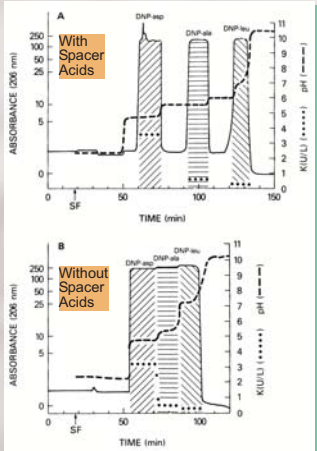
The large absorbance peak prior to the pH change contained impurities, either from the samples or the coil, while the peaks after that showed very little or no impurities. From previous attempts at achieving separation this is not a common phenomena. Rather, impurities from various sources will tend to elute immediately behind the pH change front, not with the solvent front. This could indicate some highly water-soluble impurities present in the compounds that were not detected by HPLC analysis.

The two compounds that co-eluted are both hydrochloride salts with a similar functional group in the same location on the molecule. It could be predicted that these two molecules would behave very similarly under identical conditions. The third compound to elute, another hydrochloride salt, contains an additional phenyl moiety, providing a slightly greater affinity for the organic stationary phase. The fourth molecule is not a salt, so as expected, was strongly retained in the stationary phase in which it is highly soluble. With a later elution time excellent recovery with minimal impurities was achieved.

The ability to resolve compounds by the pH zone-refining technique is dependent on the selection of an appropriate acid/base combination. The use of two or more acids or bases, (called spacer acids or spacer bases) in the stationary phase results in changes to the elution profile, separating the individual components into clear peaks instead of one broad peak (Figure 4).

Enhanced Resolution of DNP-amino Acid Mixture Using “Spacer Acids” in the Stationary Phase *

Figure 4



* Taken from "High Speed Countercurrent Chromatography, edited by Yoichiro Ito and Walter D. Conway, John Wiley & Sons, Inc., New York, NY, 1996."

Preparative scale-up of this method should yield similar results but with an expected peak broadening and possible lack of clear resolution between the two early co-eluting and the third closely eluting compounds. No difficulty should be encountered when recovering the late eluting compound, as the retention time is so great. One solution to the problem presented by the co-eluting and early eluting compounds may be to use spacer acids in the stationary phase. This method is currently being tested with different tropane alkaloids and a slight modification of the two-phase solvent system. Preparative resolution of various compounds has been easily achieved using the pH zone-refining method. One such example is the purification of ~3 grams of starting material of a polyhalogenated fluorescein as described by Yoichiro Ito using a pH zone-refining method.

Conclusion

The pH zone-refining method employed in this experiment allowed for relatively quick partial resolution of a mixture of four different tropane alkaloids with the removal of possible contaminants. This method may be developed further for complete resolution of the compounds as well as large-scale separation of different tropane alkaloids.

References

Modern Countercurrent Chromatography, ACS Symposium Series 593, edited by Walter D. Conway and Richard J. Petroski, American Chemical Society, Washington, DC, 1995.

Countercurrent Chromatography – Apparatus, Theory & Applications, Walter D. Conway, VCH Publishers, Inc., New York, NY, 1990.

High-Speed Countercurrent Chromatography, Volume 132 in Chemical Analysis: A Series of Monographs on Analytical Chemistry and Its Applications, edited by Yoichiro Ito and Walter D. Conway, Series Editor J. D. Winefordner, John Wiley & Sons, Inc., New York, NY, 1996.

Countercurrent Chromatography: Instrumentation, Solvent Selection and Some Recent Applications to Natural Product Purification, A. P. Foucault and L. Chevolut, Journal of Chromatography A, 808, 1998, pp. 32-22.

High-Speed Countercurrent Chromatography of Apple Procyanidins, Y. Shibusawa, A. Yanagida, et. al., Journal of Chromatography A, 886, 2000, pp. 65-73.

Separation and Purification of Isoflavones from Pueraria lobata by High-Speed Countercurrent Chromatography, X. Cao, Y. Tian, et. al., Journal of Chromatography A, 885, 1999, pp. 709-713.

Separation of Crude Plant Extract with High-Speed Countercurrent Chromatography for Primary Screening in Drug Discovery, J. A. Armbruster, R. P. Borris, et. al., Journal of Liquid Chromatography & Related Technologies, 24, 2001, pp. 1827-184.

Preparative Separation of Components of the Color Additive D&C Red No. 28 (phloxine B) by pH Zone-Refining Countercurrent Chromatography, A. Weisz, D. Andrzejewski, and Y. Ito, Journal of Chromatography A, 678, 1994, pp. 77-84.

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